Omega-3 polyunsaturated fatty acids confer protection against gentamicin-induced testicular injury: Novel insights into possible mechanisms

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Gentamicin (GM) is an aminoglycoside that has harmful effects on the male germ cells and sperm quality. N-3 polyunsaturated fatty acids (n-3 PUFA) are natural antioxidants that influence cell signaling and inflammation. Heme-oxygenase-1 (HO-1) and heat shock proteins (HSP) aid in cellular protection against cellular insults. This study aimed to explore the potential alleviating influences of treatment with n-3 PUFA on GM-induced testicular damage. Thirty-two albino male rats were divided into four equal groups. (1) The control group received normal saline, (2) the n-3 PUFA group received 100 mg/kg body weight/day n-3 PUFA daily for 4 weeks, (3) the GM group received 100 mg/kg/day GM intraperitoneally for 10 consecutive days, and (4) the GM + n-3 PUFA group received intraperitoneal GM for ten days followed by treatment with n-3 PUFA for 4 weeks. Significant reductions in sperm motility, viability, serum testosterone, total testicular protein, and germinal epithelium height were observed in the GM-treated group, with upregulation of the oxidative stress markers, HO-1 mRNA, and HSP70, and downregulation of proliferating cell nuclear antigen (PCNA). We also observed cellular disorganization, vacuolation, tubular distortion, and a significantly higher percentage of collagen. Ultra-structurally, most of the spermatogenic cells were electron dense and degenerated with rarefied cytoplasm. Treatment with n-3 PUFA resulted in a significant increase in sperm motility, viability, serum testosterone, and in the germinal epithelium height. Upregulation of HO-1 mRNA, HSP70, and PCNA expression and a significant reduction in the oxidative stress index were also observed. The findings confirm the potential ameliorative role of and imply novel mechanisms by which n-3 PUFA protects against GM-induced testicular injury.

Key words: Polyunsaturated fatty acids, gentamicin, oxidative stress, testis, male infertility.

INTRODUCTION

Spermatogenesis is a complex cellular process responsive to developmental factors and sensitive to...
chemical toxins and environmental stress. Oxidative stress is one of the major causes of male infertility owing to its detrimental influence in the developing germ cells and sperm quality (Aly and Khafagy 2014). Antioxidants obtained from natural sources have been employed for protection against testicular damage. N-3 polyunsaturated fatty acids (n-3 PUFA) are long chain polyunsaturated fatty acids of plant and marine origin. Three of the most well-known among them, α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are considered strong intracellular and intercellular mediators (Ambrozova, Pekarova et al. 2010). They play a fundamental role in cell metabolism, signaling, and inflammation (Attuwaybi et al. 2004, Vaughan, Garcia-Smith et al. 2012) and are capable of reducing lipid levels, blood pressure, thrombosis and reactive oxygen species (ROS) production, and the modulation of nitric oxide production (Demaison and Moreau 2002).

The transformation into biliverdin, carbon monoxide (CO), and free iron (Fe$^{2+}$) depends on the expression of heme oxygenase (HO), which is regarded as the rate limiting step in this process (Fazekas et al. 2012). Upregulation of HO-1 is one of the main cytoprotective mechanisms activated during cellular stress. Under normal homeostatic conditions, HO-1 plays a fundamental role in the physiological regulation of various organs including the testes (Lu et al. 2018). Overexpression of HO-1 has been shown to confer remarkable cellular protection in numerous in vivo and in vitro models of cellular insult (Katori et al. 2002). HO-1 has also been reported to be a powerful moderator of the upregulation of antioxidant enzymes as well as restraining both intracellular and mitochondrial ROS generation Xu et al. 2014, Gorojod et al. 2018). Additionally, it suppresses both apoptosis and the inflammatory response, maintains microcirculation, stabilizes the cell membrane, and maintains both mitochondrial fusion and fission (Zhang et al., 2013, Chi et al., 2016, Gorojod et al. 2018). Furthermore, biliverdin and CO, the biologically active products of HO-1, can reinforce its versatile functions. Biliverdin is thought to scavenge ROS and repress inflammatory reactions (Sarady-Andrews et al. 2005, Öllinger et al. 2007), while CO can promote mitochondrial biogenesis, improve blood flow, upregulate growth factors, and suppress the inflammatory response (Wang et al. 2016, Magierowski et al. 2017).

Heat shock proteins (HSP) are cytoprotective intracellular proteins. Expression of HSP increases in response to various stresses such as inflammation, ischemia, and the presence of some chemicals that repair or degrade aberrantly mutated or folded proteins (Sóti et al. 2005). HSP70, a 70 kDa HSP, has cytoprotective effects on the heart, stomach, and liver (Otake et al. 2007).

The aminoglycoside antibiotic gentamicin (GM) is widely used against a broad spectrum of microbial pathogens, particularly those that are Gram-negative (Kim et al. 2014). It has been suggested that its overuse is associated with many adverse effects on different body organs, including the structure and function of the testes. GM has been known to increase oxidative stress in the testes by enhancing free radical formation and lipid peroxidation (Zahedi et al. 2010). It has also been suggested that GM impairs sperm motility, reduces reproductive organ weight, and induces cell apoptosis in the testes, eventually resulting in testicular failure (Khaki et al. 2009, Nouri et al. 2009, Zahedi et al. 2010). While various reports have shown the injurious effects of GM on the testes (Kim et al. 2014, Aly and Hassan 2018, Cherif et al. 2019), few have evaluated its ultra-structural effects. Thus, this study was designed to investigate the potential of n-3 PUFA to alleviate GM-induced testicular damage and to provide insight into possible mechanisms.

MATERIALS AND METHODS

Animals and experimental design

Albino male Wistar rats (n=32; 120-150 g) obtained from Assiut University Animal House (Assiut, Egypt) were housed in well-aerated stainless-steel cages at room temperature (23±3°C). Control and experimental rats received a standard diet of rodent chow and water ad libitum. Rats were exposed to alternating periods of 12 h of light followed by 12 h of dark. All experiments were performed daily between 8:00 and 10:00 am. This work was approved by the Animal Ethics Committee of the Faculty of Medicine of Assiut University.

All the animals were cared for according to the guidelines provided by the Animal Ethics Committee of Assiut Faculty of Medicine and the National Institutes of Health Guidelines. After 7 days of acclimatization, the rats were randomly assigned to four groups (eight rats per group), as follows: (1) the control group received normal saline intraperitoneally (I.P.); (2) the n-3 PUFA group received n-3 PUFA (Doppelherz, Germany) daily for 4 weeks at a dose of 100 mg/kg body weight/day (Chahardahcherik et al. 2013) intragastrically using a stomach tube; (3) the GM group received GM I.P. (80 mg amouple, Memphis Pharm. & Chemical Ind., Cairo, Egypt) at dose of 100 mg/kg/day for 10 consecutive days to induce significant testicular damage (Aly and Hassan 2018); and (4) the GM + n-3 PUFA group received GM for 10 days, followed by n-3 PUFA for 4 weeks (using the same doses as those used in the n-3 PUFA and GM groups).

At the end of the experiment, fasting venous blood samples for all groups were taken from the tail veins. Blood samples were centrifuged at 3000 × g for 15 min and the resultant sera were kept at -20°C until used in the serum testosterone assay. Following blood sampling, the rats were euthanized, and the testes were immediately removed, washed in ice-cold physiological saline, dried, and weighed. The cauda epididymides from each rat were used for evaluation of sperm motility and viability. The right testis was quickly frozen in liquid nitrogen and stored at -80°C for use in preparing tissue homogenate. The testicular tissue was homogenized in ice-cold phosphate buffer (pH 7.0); the homogenates were centrifuged at 5000 × g for 15 min at 4°C and used for biochemical and molecular biology analyses. The left testis from all animals was prepared for histopathological and immunohistochemical examination.
Sperm collection

The cauda epididymis was excised, minced, and incubated in a pre-warmed Petri dish including 10 mL Hank’s balanced salt solution at 37 °C. The spermatozoa were allowed to disperse in the buffer. The suspension was shaken gently to homogenize the cells prior to analysis under a light microscope at a magnification of 400X (Carvajal et al. 2018).

Sperm evaluation

Sperm motility

Sperm motility was evaluated during the study by the same person each time using visual assessment of 100 spermatozoa per animal, in duplicate, using a phase-contrast microscope at 200X magnification. Spermatozoa were classified as immotile, motile without progression, or motile with progressive movement (Seed et al. 1996).

Sperm viability

Viability was assessed using eosin Y staining (5% in saline). 40 μL samples of the fresh sperm suspension were placed on glass slides, mixed with 10 μL eosin, and examined under a light microscope (400X magnification). Following staining, live sperm remained unstained, while dead sperm showed pink or red coloration. From each sample, at least 200 sperm were counted randomly in ten fields of vision, from which the percentage of live sperm was calculated (Björndahl et al. 2003).

Biochemical analysis

Serum testosterone

Serum testosterone was measured using the ELISA method and a commercially available kit (ALPCO, Diagnostics. NH, USA).

Testicular total protein and cholesterol

The total tissue protein and total cholesterol levels of the testes were estimated spectrophotometrically according to the methods reported by Lowry et al. (Lowry et al. 1951) and (Zlatkis et al. 1953) respectively.

Assessment of the total antioxidant capacity (TAC), total oxidant status (TOS, total peroxide concentration) and oxidative stress index (OSI)

The total antioxidant status in the testicular tissue homogenate was evaluated using a commercial kit (Biodiagnostic, Egypt) following the manufacturer’s instructions. The results are expressed as mmol/L. The total peroxide (TOS) level of the tissue samples was measured in μmol/L using a novel automated method developed by Erel (Erel 2004). Calculation of the OSI was performed using the TOS:TAC ratio. Following conversion of the unit of TAC (mmol/L) to μmol/L, the OSI was calculated as follows: OSI = [(TOS, μmol/L)/(TAC, μmol/L) × 100] (Yanik et al. 2004).

Molecular detection of HO-1 mRNA using quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was isolated from testicular tissue using a kit for total RNA extraction from tissues (Qiagen, GmbH, Germany). cDNA was synthesized using a power cDNA Synthesis Kit (INtRON Biotechnology, Inc., Korea). Real-time amplification was performed using primers selective for HO-1 and β-actin as a housekeeping gene. The primer sequences were HO-1 (forward 5’-CAGGTTGATGCTGACAGAGGA-3’ and reverse 5’-ACAGGAAGCTGAGTGGAGG-3’) and β-actin (forward: 5’-CACGATGGAGGGGCGGGACTCAT-3’ and reverse: 5’- TAAAGACCTCCTATGCAACACAGT-3’). The primers were obtained from (Invitrogen, UK). Primers were designed using the Primer-Blast program from the National Center for Biotechnology Information and reconstructed according to manufacturer’s instructions. The reaction mixture used for the qPCR amplification consisted of IQ SYBR Green Supermix (10 μL), 0.5 μL Rox, 0.5 μL HO-1 and β-actin primers, 2 μL H2O, and 6 μL cDNA. The amplification program was performed with an initial denaturation step (5 min at 95°C), followed by 40 cycles of PCR (30 s at 95, 30 s at 55°C, and 30 s extension at 72°C). The calculated cycle threshold (Ct) values were exported to Microsoft Excel to be analyzed. Ct values were normalized using β-actin as the internal standard using the Delta-Delta comparative threshold method (Livak and Schmittgen 2001).

Light microscopy (LM)

Testicular samples were fixed using 10% neutral buffered formalin for the histopathological and immunohistochemical evaluations, then processed to paraffin blocks. Sections (4 μm) were sliced and stained with Hematoxylin and Eosin (H&E), Masson’s trichrome (MT), and periodic acid Schiff’s reaction stain (PAS) to be examined using a light microscope (Olympus CH, Japan) (Bancroft and Gamble 2008).

Transmission electron microscopy (TEM)

The testicular samples were divided into pieces (2×2 mm) and processed according to methods previously described (MA 2000). The sections were examined using a Jeol-JEM-100 CXII transmission electron microscope (Jeol, Tokyo, Japan) at the Electron Microscopic Unit of Assiut University, Assiut, Egypt.

Immunohistochemistry for PCNA and HSP70

Paraffin sections were cut (4 μm) and placed on positively charged glass slides, which were then deparaffinized and rehydrated. To expose antigenic sites, the sections were incubated with 3% H2O2 for 10 min to inhibit endogenous peroxidase activity and for 20 min in 0.01 mol/L citrate buffer (pH 6.0) using microwave irradiation. To identify the nuclei of the proliferating cells, the sections were stained with a monoclonal antibody against proliferating cell nuclear antigen (PCNA; Sigma-Aldrich Inc., St. Louis, MO, USA) for 1 h at a dilution of 1/100-400; the primary antibody HSP70 (Abcam ab2787, USA) was diluted 1/100-200. The slides were washed and incubated with biotinylated secondary antibodies and the avidin-biotin complex. Finally, sections were developed with 0.05% dianinobenzidine, counterstained with haematoxylin, dehydrated,
and n-3 PUFA groups (P<0.001). n-3 PUFA administration led to significant improvement in testis weight in the GM + n-3 PUFA group compared to those of the GM-treated rats (P<0.01). Nevertheless, the testis weight of GM + n-3 PUFA rats was still significantly lower than those of rats in the control and n-3 PUFA groups (P<0.001) (Table 1).

A significant decline in sperm motility was observed in the GM-treated group compared to motility in both the control and n-3 PUFA groups (P<0.001). Also, the sperm viability was significantly reduced in the GM-treated group versus the control and n-3 PUFA groups (P<0.001). Sperm motility and viability showed significant improvement following n-3 PUFA administration in the GM + n-3 PUFA group compared to those in the GM-treated group (P<0.001). However, administration of n-3 PUFA to GM-exposed rats did not normalize the sperm motility or viability (Table 1).

**n-3 PUFA mitigated disturbances of serum testosterone, testicular total protein, and testicular total cholesterol levels induced by GM exposure**

GM-treated rats showed significantly lower serum testosterone levels compared to those of controls and n-3 PUFA treated rats (P<0.001) (Table 2). A significant elevation in serum testosterone levels was detected in GM-exposed rats after treatment with n-3 PUFA compared with those in the GM group (P<0.001). However, the serum testosterone levels in the GM + n-3 PUFA group were still significantly lower than those of the control and n-3 PUFA groups (P<0.001 and P<0.001, respectively).

According to Table 2, treatment with GM resulted in a significant decline in total testicular protein in the GM-treated group compared with that in the control and n-3 PUFA groups (P<0.001). In addition, n-3 PUFA led to a significant increase in the total protein level in the GM + n-3 PUFA group versus the GM-treated group (P<0.01). However, n-3 PUFA failed to normalize testicular total protein levels.

GM treatment caused a remarkable accumulation of cholesterol in the testes of the GM group compared with

### Table 1. Effects of gentamicin (GM) and n-3 PUFAs on testes weight and sperm characteristics of experimental groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>n-3 PUFAs</th>
<th>GM</th>
<th>GM+ n3- PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testis weight (mg)</td>
<td>1.98±0.07</td>
<td>1.92±0.1</td>
<td>0.89±0.08(^{a,b})</td>
<td>1.31±0.09(^{a,b,c})</td>
</tr>
<tr>
<td>Motility %</td>
<td>85.66±1.2</td>
<td>86±1.5</td>
<td>61±1.2(^{a,b})</td>
<td>72.33±1.2(^{a,b,c})</td>
</tr>
<tr>
<td>Viability %</td>
<td>87.69±1.4</td>
<td>87.33±1.89</td>
<td>64.33±1.6(^{a,b})</td>
<td>75.67±1.75(^{a,b,c})</td>
</tr>
</tbody>
</table>

Data were displayed as mean ± SEM of eight rats. Data were analyzed by a one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test. \(^{a}\)Significantly different vs. normal control group (P < 0.05). \(^{b}\)Significantly different vs. n-3 PUFAs group (P < 0.05). \(^{c}\)Significantly different vs. GM group (P < 0.05).

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Morphometric study of the testicular epithelium

Computerized image analysis system software (Leica Q 500 MCO; Leica, Germany) connected to a camera attached to a Leica Universal Microscope was used to evaluate the height of the tubular epithelium in the H&E stained sections of the testis in the different groups. The height of the epithelium was measured, in micrometers, in 50 randomly chosen tubules from 5 non-overlapped sections of the testes from 3 rats per group. It was measured at two points on the same axis of each tubule using an arbitrary distance method and viewing the sections with a 40X objective lens. The epithelial height was the mean of these two readings. Five non-overlapped sections of testis from 3 rats per group were used to measure the following:

1. The number of positive PCNA immunostained nuclei in 10 tubules in each section (measured by counting).
2. The area of distribution of positive reactions for the HSP70 antibody (expressed as a percentage).
3. The area of distribution of collagen fibers in Masson's trichrome-stained sections (expressed as a percentage).
4. The area of PAS-stained areas in PAS-stained sections (expressed as a percentage) (measured using image j program which is a Java-based open source image processing package).

### Statistical analysis

The software package Prism (graph pad version 3.0) was used for statistical evaluation. All values are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for multiple comparisons, followed by a post hoc Newman-Keuls test. Results were considered statistically significant at P<0.05.

### RESULTS

**n-3 PUFA alleviated reduction of testis weight and sperm characteristics induced by GM exposure**

Rats treated with GM demonstrated a significant reduction in testis weight compared to those of rats in the control

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**Note:** The above text is a continuation of the previously extracted content.
Table 2. Serum testosterone, testicular total protein and testicular total cholesterol of the experimental groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>n-3 PUFAs</th>
<th>GM</th>
<th>GM + n-3 PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum testosterone (ng/ml)</td>
<td>1.36±0.07</td>
<td>1.31±0.07</td>
<td>0.51±0.06a</td>
<td>0.91±0.06a,b,c</td>
</tr>
<tr>
<td>Testicular total protein (mg/gm tissue)</td>
<td>23.15±1.36</td>
<td>24.68±1.45</td>
<td>13.26±1.21a</td>
<td>18.35±1.15a,b,c</td>
</tr>
<tr>
<td>Testicular total cholesterol (mg/gm tissue)</td>
<td>6.15±0.52</td>
<td>5.86±0.48</td>
<td>14.15±0.63a</td>
<td>8.18±0.74a,b,c</td>
</tr>
</tbody>
</table>

Data were displayed as mean ± SEM of eight rats. Data were analyzed by a one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test. *Significantly different vs. normal control group (P < 0.05). a,b Significantly different vs. n-3 PUFAs group (P < 0.05). c Significantly different vs. GM group (P < 0.05).

Figure 1. (A) Levels of total antioxidant capacity (TAC), (B) levels of total oxidant status (TOS) and (C) oxidative stress index (OSI) in the testes tissue homogenate of the studied groups. Data were displayed as mean ± SEM of eight rats. Data were analyzed by a one-way analysis of variance (ANOVA) followed by a post hoc Newman-Keuls test. *Significantly different vs. normal control group (P < 0.05). #Significantly different vs. GM group (P < 0.05).

n-3 PUFA abrogated GM-induced oxidative stress

A significant reduction of TAC and a remarkable elevation of TOS levels and OSI were found in GM-treated animals compared to the control (P<0.001 for all) and n-3 PUFA (P<0.001 for all) groups. Unlike the GM-treated rats, the animals in the GM + n-3 PUFA group demonstrated improved TAC levels (P<0.05) and decreased TOS levels (P<0.05) and OSI (P<0.05), although these levels did not reach normal values (Figure 1).

Upregulation of HO-1 mRNA by GM and n-3 PUFA treatment

This study demonstrated a remarkable increase in the HO-1 mRNA expression level in GM-treated rats compared to the level in the control and n-3PUFA groups...
A further increase in the HO-1 mRNA expression level was observed in GM-exposed animals after treatment with n-3 PUFA when compared with the control (P<0.001), n-3 PUFA (P<0.001), and GM (P<0.05) groups (Figure 2).

**Histological results**

**Light microscopic results**

**H and E stained sections**: From control and n-3 PUFA-treated animals, it revealed that the testicular tissue was formed of rounded or oval seminiferous tubules with regular outlines and lined with stratified germinal epithelium. These tubules contained mature sperm and were separated by minimal interstitial tissue (Figure 3a and b). In the GM-treated group, the tubules were affected to a variable degree by cellular disorganization, tubular distortion, and widening of the intratubular spaces. In addition, there was a marked decline in germinal layers with vacuolation and acidophilic material in the interstitial tissue (Figure 3c). Both the GM- and n-3 PUFA-treated groups showed a reorganization of most of the tubules and arrangement of the germinal epithelium in multiple layers (Figure 3d). Morphometric results revealed a significant reduction of the germinal epithelium in the GM-treated group compared to the control group, accompanied by a significant increase in the epithelium height in the GM + n-3 PUFA group compared to that in the GM group (Figure 3e).

**When viewed using a higher magnification**: The previously viewed sections revealed normal structure in the germinal epithelium in both the control and n-3 PUFA-treated groups. This normal structure was distinguished by the presence of spermatogonia resting on the basement membrane, primary spermatocytes arranged in several layers with the largest vesicular nuclei, rounded spermatids close to the lumen, the appearance of spermatozoa in the lumen, and Sertoli cells interspersed among the germinal cells (Figure 4a and b). In the GM-treated group, the seminiferous tubules were markedly affected by cellular disorganization, deeply stained nuclei, and ill-defined spermatogenic cells with cytoplasmic vacuolation (Figure 4c). In addition, testes from the GM + n-3 PUFA group showed an improvement in most of the seminiferous tubules in the form of a rearrangement of cell layers (Figure 4d).

**Masson’s trichrome sections**: In both control and n-3 PUFA groups revealed minimal deposition of collagen fibers in the capsule and the interstitium (Figure 5a and b). In the GM-treated group, an increase in collagen deposition was found (Figure 5c). However, a decrease in collagen deposition was noticed in the GM + n-3 PUFA group after treatment with n-3 PUFA (Figure 5d). Statistically, a significant increase in collagen fibers was
detected in the GM-treated group compared to the amount seen in the control group, while a significant reduction was found in the GM + n-3 PUFA group compared to that in the GM group (Figure 5e).

**Figure 3.** Photomicrographs of H&E stained sections. (a) Control testis: showing the regular arrangement of seminiferous tubules and wholly appearance of mature sperm (arrowheads). Notice, regular and minimal interstitial tissue (*). (b) n-3 PUFAs treated testis showing regular seminiferous tubules, wholly appearance (arrowheads) and normal interstitial tissue (*). (c) GM treated testis showing marked disorganization of spermatogenic cells with the widening of intratubular spaces (arrowheads). Detachment and decrease of spermatogenic layers (↑), vacuolation in the interstitial tissue (*) can be seen. (d) GM and n-3 PUFAs treated testes showing reorganization of testicular spermatogenic cells in multiple layers (↑). The wholly appearance of spermatozoa (arrowhead) and a decrease in the interstitial tissue (*) can be seen (H&E X100). (e) Height of germinal epithelium in different groups, there is a significant decrease in the GM group and a significant increase in GM+ n-3 PUFAs treated group.

**PAS-stained sections:** Revealed minimal PAS-stained material in both the control and the n-3 PUFA groups (Figure 6a and b). The GM-treated group showed PAS-positive material in the interstitium (Figure 6c), while the
Figure 4. Higher magnifications of the previous sections. (a) Control showing regular basal lamina basement membrane (bm), normal height and arrangement of spermatogenic cells as spermatogonia (S) with small vesicular nuclei, 1ry spermatocytes (Sp) with large vesicular nuclei, Spermatid (St) and pale nuclei of Sertoli cells (↑). Interstitial cells appear vesicular (*) and surrounded with minimal C.T. (b) n-3 PUFAs group showing the regular arrangement of spermatogenic cells spermatogonia (S), primary spermatocytes (Sp), spermatid (St) with interstitial tissue (*). (c) GM testis showing; marked vacuolation (v) and distortion of spermatogenic cells. Most of the cells have degenerated; few appear shrunken with dense nuclei (arrowheads). (d) GM and n-3 PUFAs group showing rearrangement of spermatogenic cells in several layers but most of them are dark and ill-defined (*). H&E X 400.

GM + n-3 PUFA group showed minimal PAS-stained material after treatment with n-3 PUFA (Figure 6d). Statistically, the GM-treated group had a significantly higher amount of PAS-stained material than the control group. The GM + n-3 PUFA group had a significantly lower amount of PAS-stained material than the GM-treated group (Figure 6e).

Immunostained sections with PCNA: Positive immunostained (immunopositive) cells were seen in the basal layer of tubules in control testes (Figure 7a) while testes from the N-3 PUFA group showed positive immunostained cells in the different layers (Figure 7b). A decrease in immunopositive cells was noticed in the GM-treated group (Figure 7c). However, a marked increase in immunopositive cells in several layers in most tubules was detected in the GM + n-3 PUFA group after administration of n-3 PUFA (Figure 7d). Morphometric results revealed a significant decline in PCNA positive cells in the GM-treated group compared to those in controls and a significant increase in positive cells in the GM + n-3 PUFA group compared with those in the GM-treated group (Figure 7e).

Sections immunostained for HSP70: Revealed a mild reaction mainly in the basement membrane in both the control and n-3 PUFA groups (Figure 8a and b). A moderate reaction to HSP70 was found in the GM-treated group (Figure 8c). In addition, a marked reaction to HSP70 was observed in the GM + n-3 PUFA group following treatment with n-3 PUFA (Figure 8d). Statistically, the GM-treated and GM + n-3 PUFA groups had a significantly higher reaction to HSP70 than the controls (Figure 8e).

Electron microscopy results

Electron microscopic examination of the control and n-3 PUFA groups showed spermatogonia resting on the basement membrane with its ultra-structure in the form of rounded nuclei containing clumps of heterochromatin and
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Figure 5. Photomicrographs of Masson’s trichrome stained sections. (a) Control testis showing regular distribution of collagen fibers as a thin testicular capsule (C) and interstitial tissue (I). (b) n-3 PUFAs testis showing thin capsule (C) and interstitial tissue (I). (c) GM treated testis showing an increase in collagen fiber deposition in the testicular capsule (C) and in the interstitial tissue (I). (d) GM and n-3 PUFAs treated testis showing: decrease in the deposition of collagen fibers in the testicular capsule (C) and in the interstitial tissue (I) (Masson’s trichrome X100). (e) Showing collagen area % in different groups.

scanty electron-dense cytoplasm containing mitochondria and ribosomes (Figure 9a). Primary spermatocytes were identified by their large rounded nuclei with dispersed chromatin and the large area of cytoplasm with mitochondria and ribosomes (Figure 9a).

The interstitial cells of Leydig could be recognized by their large rounded nuclei with cytoplasm containing parallel cisternae of rough endoplasmic reticulum (rER), ribosomes, and multiple mitochondria (Figure 9b). In the GM-treated group, degenerative changes were noticed in spermatogonia in the form of electron-dense degenerated cytoplasm with damaged mitochondria (Figure 9c). In addition, primary spermatocytes were seen with defined rounded nuclei and ill-defined degenerated cytoplasm (Figure 9d) and vacuoles and damaged mitochondria were seen in the cytoplasm of the Leydig cells (Figure 9e).

In the GM + n-3 PUFA group, treatment with n-3 PUFA was followed by an improvement in the degenerative changes as the ultra-structure of the spermatogonia and primary spermatocytes were more or less similar to that of the controls (Figure 9f). The Leydig cells also showed...
rounded nuclei with cytoplasm containing multiple mitochondria and ribosomes (Figure 9g).

**DISCUSSION**

Several environmental factors, including misuse of antibiotic drugs, have been implicated in testicular damage or impaired sperm function. This has turned attention towards the use of natural products as protective agents for the remediation of reproductive toxicity.

Oxidative stress plays a fundamental role in the progress of GM-induced testicular damage. In our study, GM treatment elicited a state of oxidant/antioxidant imbalance as evidenced by increased TOS levels and OSI and a reduction in the TAC level. In addition, examination using light and transmission electron
microscopy showed significant decreases in the germinal epithelium height and testicular weight, which confirms the depletion and degeneration of germ cells. These results were consistent with previous studies that reported an elevation in lipid peroxidation and an inhibition in antioxidant enzymes in the testes of GM-treated animals (Kim et al. 2014; Aly and Hassan 2018, Cherif et al. 2019). This can be attributed to GM-generated free radicals, which can affect the unsaturated fatty acids in the plasma membrane of male germ cells, resulting in elevation of lipid peroxidation and damage to the plasma membrane (Lenzi et al. 2002, Aly and Hassan).
Figure 8. HSP70 immunostained sections. (a) Control testis showing mild staining for HSP70 mainly in the basement membrane b.m. (curved arrow). (b) n-3 PUFAs group showing mild staining for HSP70 (curved arrow). (c) GM treated testis showing moderate staining for HSP70 which include basement membrane (b.m.) curved arrow, and primary spermatocytes (†). (d) GM and n-3 PUFAs treated testis showing strong staining for HSP70, basement membrane (curved arrow), spermatocytes (†) and sperms (arrowhead) ×1000. (e) Showing HSP70 area % in different groups.

2018). Also, the testes from GM-treated rats are more vulnerable to ROS damage due to the inadequacy of antioxidant enzymes, as observed by Karaman et al. (Karaman et al. 2018), who reported that GM treatment may suppress the expression of antioxidant enzyme genes.

The results presented here showed that n-3 PUFA could effectively abrogate GM-induced oxidative damage and lipid peroxidation in testicular tissues through overall alleviation of oxidative stress and by increasing antioxidant activity and depressing lipid peroxidation. Proof of this effect of n-3 PUFA was demonstrated by an improvement in most of the degenerative changes in almost all seminiferous tubules and the significant increase in the height of the germinal epithelium. n-3 PUFA supplementation has been reported to increase
Figure 9. Electron micrographs. (a) Control testis showing spermatogonia (S) resting on the basement membrane (b.m) with oval nucleus (N1), mitochondria (m) and ribosomes (r). Primary spermatocytes with large rounded nuclei (N2). (b) A Leydig cell of control group showing oval nucleus (N), mitochondria (m), free ribosomes (r) and rER. (c) GM treated testes showing Spermatogonia with folded nucleus (N1) and electron-dense degenerated cytoplasm which contains damaged mitochondria (m). The adjacent cell with a defined nucleus with (N2) and degenerated cytoplasm (*). (d) Showing two primary spermatocytes with large nuclei (N) and ill-defined degenerated cytoplasm (*), remnants of degenerated cells(curved arrow). (e) The Leydig cell of gentamicin treated group showing oval nucleus (N). The cytoplasm contains damaged mitochondria (m), and vacuoles (v). (f) GM + n-3 PUFAs treated testes showing spermatogonia (S) with rounded nuclei (N1) resting on basement membrane (b.m.) with mitochondria (m) and ribosomes(r). Two primary spermatocytes (Sp) with large euchromatic nuclei(N2). (g) Showing Leydig cell with large oval nucleus (N) and cytoplasm contains multiple ribosomes(r) and mitochondria (m) ×4800.
mitochondrial biosynthesis and its oxidative capacity (Vaughan et al. 2012). Additionally, DHA, an essential n-3 PUFA, was reported to display an antioxidant effect by improving mitochondrial dynamics, upregulation of mitochondrial fusion-related protein, and downregulation of the mitochondrial fission-related protein (Zhang et al. 2018). Furthermore, n-3 PUFA can induce protective effects against oxidative stress by modulating cytosolic Ca^{2+} release and antioxidant levels (Ateşsahin et al. 2006). This can also be attributed to the possible direct integration of n-3 PUFA into the cell membrane and subsequent stabilization of the membrane structure, as well as reduction of ROS production and lipid peroxidation (Uygur et al. 2014). In the same manner, Tatsumi et al. (Tatsumi, Kato et al. 2019) demonstrated that n-3 PUFA can exert its antioxidant effects through stimulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) translocation from the cytoplasm into the nucleus where it can stimulate the expression of antioxidant enzymes.

This provides evidence that reduced levels of lipid peroxides and the improvement of antioxidant activity may corroborate the beneficial role of n-3 PUFA as a promising antioxidant against GM toxicity in the testis. In this study, significantly upregulated mRNA expression of the HO-1 enzyme was observed in GM-treated rats in addition to an increase in HO-1 expression following supplementation with n-3 PUFA. It has been suggested that GM treatment induces a state of stress in cells by creating an inflammatory environment that may encourage expression of the HO-1 protein as a stress response (Duarte et al. 2016, Aycan-Ustytol et al. 2017, Park et al. 2017). On the other hand, recent in vivo and in vitro studies postulated the ability of n-3 PUFA to induce HO-1 expression through direct activation of its upstream Nrf2 regulator gene (Qi et al. 2017, Sakai et al. 2017, Tatsumi et al. 2019, Xu et al. 2018). However, it seems that, while Nrf2 may affect HO-1 expression, HO-1 may also regulate Nrf2 expression, as demonstrated by Chi et al. (Chi et al. 2016) observed that increased HO-1 promoted Nrf2 protein expression, while repression of HO-1 diminished Nrf2 expression, from which they suggested that HO-1 may regulate Nrf2 in a positive feedback manner.

With respect to the role of testosterone as the principal hormone responsible for male reproductive function, this study revealed that the serum testosterone level was reduced following GM treatment, which was accompanied by vacuolation, degeneration of interstitial cells, and interstitial tissue exudation. These changes were mostly attributed to the direct effect of GM on the testicular tissue, especially on the Leydig cells, and the alteration effect on the lipid profile (El-Sweedy et al. 2007). These findings are in agreement with previous observations (Ghosh and Dasgupta 1999, Carageorgiou et al. 2005, Aly and Hassan 2018). Also, it has been reported that treatment with GM reduced the rate-limiting enzymes of testosterone synthesis, resulting in an impediment to their production (Ghosh and Dasgupta 1999). In addition, lipid peroxidation and ROS generated by GM treatment may impair testosterone production by the Leydig cells (Chen et al. 2008). This impaired testosterone synthesis could lead to cholesterol accumulation, which was observed in the GM-treated animals in the present study. It is worth mentioning that cholesterol is the fundamental substrate for testosterone synthesis in the body (Shirakawa et al. 2006). GM has been reported to suppress those steroidogenic enzymes that act on cholesterol to synthesize testosterone, which could explain the present results (Ghosh and Dasgupta 1999).

The present investigation showed that the n-3 PUFA group exhibited an increase in testosterone levels. This was in agreement with the findings of other studies (Mohammad et al. 2015, Wang et al. 2018) that reported the ability of n-3 PUFA to regulate the expression of the enzymes key for testosterone synthesis and hence the enhancement of testosterone levels. The reduced testicular cholesterol levels following supplementation with n-3 PUFA reported in this study could result from upregulation of testosterone levels.

It is well known that testosterone is the hormone most responsible for sperm production. In this study, testicle weight and sperm motility and viability were reduced in GM-treated rats. These results were corroborated by the histopathological findings of damaged testicular structure, tubular atrophy, increased apoptotic cells, inhibition of cell division, and germ cell loss. Indeed, GM treatment in humans and in rats may lead to a decrease in protein synthesis in the nuclei of spermatagonia and spermatocytes, resulting in cessation of spermatogonial division, interruption in primary spermatocyte meiosis, and a significant increase in the percentage of fragmented DNA in the testicular tissue (Mohamed SM1 2017). According to (Aly and Hassan 2018), decreased testosterone biosynthesis and/or bioavailability affects the character of the sperm and causes as decline in testes weight. In the present study, these findings were confirmed by a significant reduction in the number of PCNA immunostained cells in the GM-treated group compared to those of the control, which were observed in only a few basal germ cells and indicated reduced proliferative activity. High levels of ROS in sperm has been suggested to result in DNA fragmentation and chromatin cross-linking, leading to low quality sperm and fertilization problems (Meseguer, et al. 2008).

However, after administration of n-3 PUFA, there was an increase in the expression of PCNA positive cells. This improvement reflected the anti-apoptotic and proliferative function of n-3 PUFA, in agreement with previous results (Uygur et al. 2014), which demonstrated an increased proliferative activity in the spermatogenic cells in rat testes. Also, the present results reported an
increase in testis weight and an improvement in sperm motility and viability following supplementation with n-3 PUFA that can be attributed to increased testosterone levels as demonstrated by current and previous studies (Uygur et al. 2014, Khavarimehr et al. 2017, Wang et al. 2018).

Chronic treatment with GM has been known to increase the percentage of collagen fibers, especially in the capsule and interstitial tissue, with an accumulation of acidophilic fluids and vacuolation in the interstitial spaces. This leads to interstitial edema (Kim et al. 2014) with subsequent oozing of lymphatic exudates due to increased vascular permeability or interstitial degeneration. Interestingly, the aforementioned results showed the deposition of fewer collagen fibers in the interstitial tissue following n-3 PUFA treatment. These changes could be attributed to the powerful anti-inflammatory properties of n-3 PUFA that led to the inhibition of lipid peroxidation and the removal of free radicals (Olutope et al. 2014). The anti-inflammatory activity of n-3 PUFA was mediated by antagonizing the activity of arachidonic acid, which suppressed the cell-mediated immune response and leukocytic activity (Taati et al. 2011). The powerful fibrous deposition antagonizing the activity of omega-3 has been documented previously in pulmonary and cardiac tissues (Chen et al. 2011), which was due to downregulation of profibrogenic genes or changes in the composition of the cell membrane with subsequent decreased inflammation and fibrosis (Zhao et al. 2014).

Regarding HSP70, the aforementioned results demonstrated higher expression of HSP70 in the GM-treated group compared to the control as a protection against injury to the testicular tissue. Excessive production of HSP70 can definitely be considered an important response to the damage caused to spermatozonal DNA by free radicals that results in male infertility (Erata et al. 2008). Also, levels of HSP70 are markedly increased in several conditions, such as ischemia/reperfusion, acidosis, hypoxia, and oxidative stress (Kiang and Tsokos 1998). In addition, a significant induction of the percentage of HSP70 was revealed after administration of n-3 PUFA in GM-treated animals, which aided in the protection against testicular damage.

Induction of HSP70 may be attributed to either the stability of HSP70 mRNA or to an increase in the rate of transcription. The cytoprotective effect of HSP70 is explained mainly by direct binding to damaged protein, resulting in its refolding and regeneration in different germinal cells (El Golli-Bennour and Bacha 2011). In addition, HSP70 is considered to be an important antiapoptotic factor that may protect different types of cells against many apoptotic stimuli such as anticancer drugs, radiation, and oxidative stress (Samali and Orrenius 1998). We observed this antiapoptotic activity in our light and electron microscopy results, which were manifested by regeneration of spermatogenic cells and restoration of normal morphology. This anti-apoptotic activity can result either from inhibition of excessive testicular nitric oxide production mediated apoptosis through mitochondrial changes (Gotoh et al. 2004) or from blocking the intrinsic pathway by inhibition of cytochrome release.

The present data demonstrate the reduced total testis protein in GM-treated animals. This result concurs with that of (Sundin et al.), who reported that exposure to GM may result in the mistranslation of amino acids by ribosomes and can also block protein synthesis at the level of the Golgi apparatus, endoplasmic reticulum, or cell cytoplasm. Moreover, administration of n-3 PUFA exposed the animals to more protein expression owing to enhancement of insulin sensitivity (Gingras et al. 2007). It may also be related to the upregulation of signaling pathway activities associated with protein synthesis and downregulation of those involved with protein degradation (Marzuca-Nassr et al. 2016).

Conclusion

The present results provide clear evidence of the hazardous effects of GM treatment on the testis, as shown by the enhancement of oxidative stress, upregulation of expression of HO-1 and HSP70, downregulation of PCNA, decreased production of testosterone and protein, and increased total cholesterol levels. In addition, GM treatment was associated with impaired spermatogenesis as demonstrated by the decline in sperm characteristics and histopathological examination. These parameters were restored following supplementation with n-3 PUFA. For these reasons, n-3 PUFA may play a role in protecting against GM misuse and the results of this study support its effectiveness when used as a treatment strategy in dealing with testicular damage. However, further studies are warranted.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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